Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Invertebrate Pathology 84 (2003) 104–113
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Effects of long-term storage on the stability of *Op*MNPV DNA contained in TM Biocontrol-1

C. Reed,[a] I.S. Otvos,[a,*] R. Reardon,[b] I. Ragenovich,[c] and H.L. Williams[a]

[a] *Natural Resources Canada, Pacific Forestry Centre, Victoria, BC, Canada V8Z 1M5*
[b] *Forest Health Technology Enterprise Team, USDA Forest Service, Northeastern Area State and Private Forestry, 180 Canfield St., Morgantown, WV 26505, USA*
[c] *USDA Forest Service, Pacific Northwest Region, P.O. Box 3623, Portland, OR 97204, USA*

Received 27 June 2003; accepted 6 August 2003

Abstract

Orgyia pseudotsugata multicapsid nucleopolyhedrovirus (*Op*MNPV) DNA was extracted from samples representing 10 lots of TM Biocontrol-1 stored at −10 °C for 5–15 years and digested with the restriction enzymes *Bgl*II, *Pst*I, and *Sal*I. DNA from the *Op*MNPV virus strain (MEM-75-STANDARD) used to produce the TM Biocontrol-1 lots was also extracted and digested. No restriction fragment length polymorphisms were observed in any of the samples and there was no evidence of DNA degradation. This indicates that long-term cold storage of TM Biocontrol-1 had no adverse effect on the quality of the *Op*MNPV DNA. In addition to the expected >23 kb *Op*MNPV DNA, extracts from lots 4a, 5b, and 6 contained 10 additional nucleic acid segments, ranging in size from 0.9 to 4.2 kb. The electrophoretic profile of these segments was characteristic of *O. pseudotsugata* cypovirus (*Op*CPV). RNase A/DNase I treatment showed that the nucleic acid contaminants were composed of RNA, suggesting that lots 4a, 5b, and 6 contained *Op*CPV as well as *Op*MNPV. Bioassay results have shown that there is a decrease in efficacy of stored TM-biocontrol-1, but this did not appear to be directly correlated with the length of time in storage.

Keywords: *Orgyia pseudotsugata*; Nucleopolyhedrovirus; Biological insecticide; TM Biocontrol-1; Long-term storage; *Op*MNPV DNA

1. Introduction

TM Biocontrol-1 is a biological insecticide that was produced and registered by the United States Department of Agriculture (USDA) Forest Service for the purpose of suppressing outbreaks of Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough). TM Biocontrol-1 contains the active ingredient *Op*MNPV, a naturally occurring nucleopolyhedrovirus effective in controlling *O. pseudotsugata* populations because it is highly pathogenic to *O. pseudotsugata* larvae (Hughes, 1976; Hughes and Addison, 1970; Martignoni, 1999).

To propagate *O. pseudotsugata* virus, host insects free of endogenous viral infections are required. An inbred colony of virus-free *O. pseudotsugata*, called the GL-1 strain, was established from eggs collected in 1965 near Goose Lake in northern California (Martignoni, 1999). This colony was maintained at the USDA Forest Service Pacific Northwest Research Station, Forestry Sciences Laboratory, in Corvallis, Oregon until 1994 (Hadfield and Magelssen, 1995), and at the Pacific Forestry Centre, Canadian Forest Service, Natural Resources Canada, in Victoria, BC since late 1994. From 1980 to 1992, *O. pseudotsugata* virus was propagated in late instar GL-1 larvae at the Corvallis Forestry Sciences Laboratory. The virus-killed larvae were frozen and sent to companies for processing to recover virus from the dead larvae and thus produce TM Biocontrol-1. The first lot was packaged in 1985 and the last lot was packaged in January 1995 (Hadfield and Magelssen, 1995). The exclusive source of virus for all of the production lots of TM Biocontrol-1 was a strain of the multinucleocapsid *O. pseudotsugata* NPV (*Op*MNPV) called MEM-75-STANDARD, which was established by the USDA

* Corresponding author. Fax: +250-363-0775.
E-mail address: iotvos@pfc.forestry.ca (I.S. Otvos).

0022-2011/$ - see front matter © 2003 Published by Elsevier Inc.
doi:10.1016/j.jip.2003.08.002

Forest Service in 1978. The propagation and amplification of *Op*MNPV followed the "seed-lot system" used by Fox et al. (1943) for the production of a strain of yellow fever virus. This system was implemented to avoid contamination of the pathogen, to ensure consistency among lots, and to minimize the chance of mutation of the virus. No more than two passages of virus separated the produced lots from the primary "seed lot" of the MEM-75-STANDARD strain.

Production of TM Biocontrol-1 from infected *O. pseudotsugata* larvae required a method that concentrated the viral inclusion bodies and excluded most of the unwanted insect debris. Larval cadavers were homogenized in water and the slurry was centrifuged to concentrate the polyhedral inclusion bodies (PIBs). The virus-rich sediments were lyophilized, screened to a fine powder, and vacuum-packaged for long-term storage (Martignoni, 1999). Although the USDA Forest Service could rear large numbers of infected larvae, they did not possess the equipment necessary to process large quantities of the final TM Biocontrol-1 product. In 1983, the USDA-Agricultural Research Service processed the first batch of TM Biocontrol-1 from 18 kg of infected larvae. A portion of this batch was used for research, and the remainder, 1680 acre-doses (representing lot 1), was returned to the USDA Forest Service. When the cooperative project was initiated in 1998, only 480 acre-doses of lot 1, which had been in cold storage the longest (since 1985), remained (Hadfield and Magelssen, 1995).

Large scale processing of TM Biocontrol-1 was subsequently contracted out to three different companies (Table 1). The first contract awarded by the USDA Forest Service was to Reuter Laboratories in 1986, and this company processed 147,600 acre-doses (lots 2, 3, 4, and 5). The next contract was awarded to Espro in 1988, and this company processed 170,566 acre-doses (lots 6 and 7). In 1990 Espro was acquired by Crop Genetics and this company continued the processing under contract for the USDA Forest Service. Crop Genetics produced 108,784 acre-doses (lots 8, 9, and 10). Virus production ceased in 1992 after an adequate supply of TM Biocontrol-1 had been produced and stored (Hadfield and Magelssen, 1995).

Table 1
Amounts and storage dates of TM Biocontrol-1 processed from 1985 to 1992[a]

Lot No.	Processing company	Amount (acre-doses)	Date stored
1	Agri. Res. Service	480	1985
2	Reuter Labs	18,000	1986
3	Reuter Labs	13,000	1986
4a	Reuter Labs	24,800	1986
4b	Reuter Labs	33,000	1986
5a	Reuter Labs	10,800	1986
5b	Reuter Labs	26,000	1986
6	Espro	17,383	1989
7a	Espro	48,300	1990
7b	Espro	50,000	1990
7c	Espro	50,000	1990
8a	Crop Genetics	690	1991
8b	Crop Genetics	68,000	1991
9b	Crop Genetics	5784	1993
9c	Crop Genetics	9000	1993
10a	Crop Genetics	1000	1995
10b	Crop Genetics	24,000	1995

[a] Information taken from Hadfield and Magelssen (1995).

The purpose of this investigation was to examine the stability of the *Op*MNPV genomic structure contained within the stored TM Biocontrol-1 samples. Efficacy of these samples is also examined.

2. Materials and methods

2.1. Extraction of OpMNPV and OpCPV from TM Biocontrol-1

Seventeen samples of TM Biocontrol-1 obtained from the USDA Forest Service for an efficacy study (Kukan et al., 2001) were used as a source of *Op*MNPV DNA. These samples represented the different package sizes (one to three) produced for each of the 10 lots of TM Biocontrol-1 processed and stored from 1985 through 1995 (Table 1), with the exception of one package (8a) that contained less than 700 total acre-doses. The four lots and 13 sub-lots examined in this study (Table 1) cover almost the entire range of time that TM Biocontrol-1 was held in cold storage (5–14 years).

To extract *Op*MNPV DNA, 0.5–1.0 g of each TM Biocontrol-1 sample was suspended in 20 ml of sterile distilled water. The suspension was centrifuged at 2000*g* for 10 min. The supernatant was removed and the pellet resuspended in 20 ml of sterile distilled water, centrifuged at 1300*g* for 10 min, and the supernatant discarded. The upper white layer of the pellet was carefully suspended in 1.5 ml of sterile distilled water and transferred to a microfuge tube. Samples were centrifuged at 14,000*g* for 15 min to pellet the polyhedral inclusion bodies (PIBs). The pellet was washed three times with sterile TE (10 mM Tris–HCl, pH 7.6; 1 mM EDTA, pH 8.0). The final pellet was resuspended in 0.1 M Na_2CO_3 and incubated at room temperature for 30 min to dissolve the polyhedra. Samples were centrifuged at 1000*g* for 5 min to pellet any large debris, and the supernatant, containing the virions, was transferred to a fresh microfuge tube and centrifuged at 18,000*g* for 30 min. The pellet was suspended in 500 μl of TES (100 mM Tris–HCl, pH 7.5; 1 mM EDTA, pH 8; and 0.1% SDS), 200 μg/ml proteinase K was added, and the samples were incubated overnight at 55 °C. Proteins were separated from viral DNA with two phenol:chloroform:isoamyl alcohol (25:24:1) extractions, and the DNA was further purified and concentrated using the GENECLEAN kit (Qbiogene). One microliter of each sample was run on a 0.6% agarose gel containing 0.2% Synergel (Diversified Biotech) stained with 100 ng/ml ethidium bromide. Genomic *Op*MNPV DNA was isolated from each production lot and digested (cut) with a series of restriction enzymes. The resulting *Op*MNPV genomic profiles were compared to profiles of MEM-75-STANDARD DNA and *O. pseudotsugata* larval host DNA digested in the same manner. DNA concentrations were estimated by comparing the sample band intensities to those of 125 ng of Lambda DNA digested with *Hin*dIII.

2.2. Extraction of OpMNPV from infected O. pseudotsugata larvae

MEM-75-STANDARD was provided by the USDA Forest Service for comparison with the samples from the stored product. To obtain enough sample of MEM-75-STANDARD to use as a positive control, newly molted third instar *O. pseudotsugata* larvae (Goose Lake strain) were inoculated with a dilute sample of MEM-75-STANDARD (75-F-06). This single passage of the standard allowed for suitable amplification of the original form of the virus while at the same time minimizing the amount of valuable MEM-75-STANDARD used. The concentration of PIBs in MEM-75-F-06 was calculated using a hemocytometer and 5 μl aliquots of the stock solution. The number of PIBs was determined to be 1.63×10^7 PIBs/ml. Ten microliters (16,300 PIBs) of a 1:10 dilution of the MEM-75-F-06 stock was applied evenly to the top of each artificial diet cube (6 mm × 6 mm × 3 mm) and allowed to soak in. The diet cube was placed in a 150 mm × 15 mm petri dish (Fisher Scientific) containing 10 fifth instar *O. pseudotsugata* larvae. When required, the cube was rehydrated with 10 μl of dH_2O to ensure that the larvae consumed as much of the virus contaminated diet cube as possible. Insects were reared until death, at which point the petri dishes with the cadavers were stored at −20 °C until required.

The stored frozen dead larvae were ground to a fine powder in a liquid nitrogen-cooled mortar and pestle with 0.5 ml STE-C buffer (10 mM Tris–HCl; 1 mM EDTA, pH 8.0; 50 mM NaCl; and 10 mM cysteine). The powder was transferred to microfuge tubes containing 1.0 ml of STE-C with 0.1% SDS and 0.06 mg/ml DNase I, and the tubes were incubated with gentle shaking at room temperature for 20 min. The samples were centrifuged at 150*g* for 3 min to pellet insect debris. The supernatant was transferred to a fresh microfuge tube and centrifuged at 18,000*g* for 15 min to pellet the PIBs. The pellet was washed three times with TE buffer (pH 7.6), and the final pellet was resuspended in 500 μl of 0.1 M Na_2CO_3 and incubated with gentle shaking at room temperature for 30 min. The solution was centrifuged at 18,000*g* for 30 min, and the pellet was suspended in 500 μl of TES with 200 μg/ml proteinase K and incubated overnight at 55 °C. Viral DNA was purified, concentrated, and its concentration estimated as described for the isolation of viral DNA from TM Biocontrol-1.

2.3. Extraction of host DNA

Orgyia pseudotsugata egg masses, free of *Op*MNPV, were obtained from the laboratory colony of Goose Lake strain (GL-1) and reared on virus-free artificial diet (Thompson and Peterson, 1978) at the Pacific Forestry Centre since late 1994. Insects were reared until third instar, at which point they were removed from the growth chamber and stored at −20 °C. These third instar larvae were used for the extraction of non-infected *O. pseudotsugata* DNA.

Frozen larvae were ground to a fine powder with a liquid nitrogen-cooled mortar and pestle. One milliliter of lysis buffer (10 mM Tris–HCl, pH 8.5; 5.0 mM EDTA; 0.2% SDS; 200 mM NaCl; and 200 μg/ml proteinase K) was added to each powdered larva and the slurry incubated for several hours at 55 °C. Following incubation, large debris were removed by a brief centrifugation at 150*g* for 3 min. DNA was purified from the supernatant using two phenol:chloroform:isoamyl alcohol (25:24:1) extractions. The DNA was further purified and concentrated using the GENECLEAN kit (Qbiogene), and the DNA concentration was estimated as described above.

2.4. Digestion of viral extracts and preparation of Southern blots

*Op*MNPV DNA extracted from the samples of TM Biocontrol-1, total genomic *O. pseudotsugata* DNA, and MEM-75-F-06 viral DNA were compared using restriction analysis. Twenty to fifty nanograms of DNA was digested with each of the restriction enzymes *Bgl*II, *Pst*I, and *Sal*I, (NEB) overnight at 37 °C. The digests were run in a 15 cm × 20 cm 0.6% agarose/1× TAE gel containing 0.2% synergel (diversified biotech) and 100 ng/ml ethidium bromide at 16 mA for 15 h. Digested DNA was passively transferred to nylon membranes (Hybond-N^+) according to the manufacturer's protocol (Amersham Biosciences).

2.5. Preparation of digoxigenin-labeled NPV probe

*Op*MNPV genomic DNA was extracted from TM Biocontrol-1 as described above. One hundred nanograms of DNA was heat-denatured by incubation at 94 °C for 10 min and immediately cooled on ice. To synthesize the DIG-labeled probe, denatured DNA was mixed with 151.6 μg/ml random hexanucleotides, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, and

0.35 mM DIG-dUTP. Two units of labeling grade Klenow (DNA Polymerase I) was added and the reaction mixture brought to a volume of 20 μl with sterile distilled water and incubated overnight at 37 °C. Quantification of labeled probe was conducted according to the colorimetric detection protocol included in the DIG Labeling and Detection kit (Roche Applied Science).

2.6. Chemiluminescent detection of Southern blots

Southern blots were prehybridized for 2 h at 65 °C in standard hybridization buffer (5× SSC; 0.1% *N*-lauroylsarcosine; 0.02% SDS; and 1% blocking reagent; Roche Applied Science). Digoxigenin-labeled genomic *Op*MNPV DNA was denatured for 10 min in a boiling water bath, immediately cooled in an ice bath, and 10 ng/ml was added to fresh hybridization solution. Prehybridization solution was removed from the tubes and 20 ml of hybridization solution was used for each 120 cm^2 blot. Blots were hybridized overnight at 65 °C. Following hybridization, the solution containing the probe was transferred to a fresh 50 ml tube and stored at −20 °C for use in subsequent hybridization reactions. The blots were washed twice at room temperature in 2× SSC, 0.1% SDS for 15 min each wash, then twice at room temperature in 0.1× SSC, 0.1% SDS for 30 min each wash. Detection of the digoxigenin-labeled DNA was conducted according to the manufacturer's protocol (Roche Applied Science) and visualized with exposure to Bio-Max X-ray film (Eastman Kodak).

2.7. RNase A digestion of CPV samples

To confirm that the contaminant nucleic acids detected in lots 4a, 5b, and 6 were RNA, the DNA extracts from these samples were subjected to RNase A (Sigma) or DNase I (Sigma) digestion. Replicates of CPV-containing *Op*MNPV DNA extract were digested with 1 U/ml of RNase A, 1 U/ml DNA or distilled H_2O for 20 min at 37 °C. Digests were run through a 0.6% agarose/0.2% synergel gel containing 100 ng/ml ethidium bromide. Results were visualized by UV illumination and photographed.

2.8. Bioassay

Bioassays were conducted on samples from the 10 lots of TM-Biocontrol-1 stored for 5–14 years and on freshly produced *Op*MNPV to determine if storage affected the efficacy of the virus product. Bioassays on samples from three lots (4, 7, and 10) representing 14, 10, and 5 years of cold storage (−10 °C) are described here. Results of the remaining 40+ bioassays, conducted over 3 years, will be reported in detail elsewhere.

Efficacy was measured as the amount of virus preparation of the stored product and fresh virus that caused 50% mortality (LD_{50}) of the test larvae at 14 days after exposure to the virus.

2.8.1. Virus preparation

Virus concentrations were selected to give approximately 30, 35, 45, 70, and 75% mortality as recommended by Robertson and Preisler (1992). A fresh batch of *Op*MNPV served as the positive control and a distilled water treatment served as the negative control. The fresh *Op*MNPV virus sample was prepared prior to the bioassay by homogenizing 100 virus-killed DFTM larvae in 5 ml of distilled water from an inoculated group of Goose Lake DFTM larvae with the stored *Op*MNPV virus samples from lot 1.

2.8.2. Inoculation

Larvae were inoculated using the diet plug method (Kaupp and Ebling, 1990). Either 1 μl of each viral concentration or distilled water was added to a small (3–4 mg) diet plug in each well of a 24-well tissue culture plate. The bioassays of the five viral dilutions of the three stored lots of TM Biocontrol-1, plus a fresh preparation of *Op*MNPV and distilled water were replicated three times.

2.8.3. Test larvae

The larvae came from surface sterilized *O. pseudotsugata* egg masses (Thompson and Peterson, 1978) of the Goose Lake laboratory strain. Emerging larvae were reared in groups of 10 on artificial diet (Thompson and Peterson, 1978) at 25 ± 1 °C, 50–60% RH, 16L:8D. To standardize the age of the test larvae, newly molted third instar larvae (<24 h old) were used and the larvae were starved for 16–20 h prior to the bioassay.

2.8.4. Bioassay

Forty-eight freshly molted third instar larvae were used for each virus concentration and the controls. In each replicate 1488 larvae [48 larvae × 5 dilutions × (5 virus samples + 1 positive control) + 1 negative control]. Replicates were inoculated on three consecutive days. Immediately after inoculation of the diet plug, one newly molted third instar larva was placed into each well of a 24-well tissue culture plate to feed on the treated diet plug. These larvae were held in darkness for 24 h at 25 ± 1 °C, 50–60% RH. Only larvae that consumed the entire diet plug were used in the bioassay (the rest were discarded) and were placed in individual cups (Solo P100, Solo Cup Urbana, Illinois 61801-2895) with fresh untreated diet and reared at the conditions above for 21 days. Control and viral infected larvae were reared under the same conditions in separate growth chambers to avoid cross-infection.

Larval mortality was recorded daily and the diet was changed weekly, or more often if it dried out. Only larvae that died from *Op*MNPV infection, verified by microscopic examination, were included in the analysis.

2.8.5. Data analysis

From the larval mortality data PROC PROBIT analyses (SAS Institute, 1989–1996) was used to calculate dosage mortality curves and LD_{50} values in PIBs with associated 95% fiducial limits. The fiducial limits are indicated by error bars. The larval mortality data (LD_{50}) of each replicate at day 14 and day 21 were calculated to determine if lethal dose changed over time. Because there was no statistically significant difference between the replicates all of the replicates were combined. Again there were no statistical difference between mortality at day 14 and day 21, and we therefore chose day 14 for comparison. The LD_{50} values were compared for significant differences, indicated by no overlap of the 95% fiducial limits. Because no differences were found among the replications, data were combined to estimate LD_{50} for the fresh *Op*MNPV.

3. Results

3.1. Physical properties of TM Biocontrol-1

The various lots of TM Biocontrol-1 differed quite markedly in their physical characteristics. While these differences are not likely to affect the efficacy of the product, they were noted nonetheless.

Lot 1, produced by the USDA Forest Service, was very coarse. It appeared to be a rough mixture of relatively large insect debris intermixed with a more homogeneous coarse powder. This product was so coarse that it was necessary to grind it with a mortar and pestle in order to facilitate adequate sedimentation of the PIBs for extraction of the viral DNA.

The lots produced by Reuter Labs, lot numbers 2 through 5, appeared fairly homogeneous in texture. Large insect debris was not readily apparent, and the powdered component was finer than lot 1. Samples from lot numbers 2 through 5 did not require additional grinding with a mortar and pestle in order to extract viral PIBs, although additional grinding did aid in the recovery of PIBs.

Lot numbers 6 through 10, processed by Espro/Crop Genetics, were very finely ground, and no large insect debris was apparent. Samples from these lots required no additional grinding with a mortar and pestle to aid in PIB recovery.

Extraction of viral DNA from the various samples of TM Biocontrol-1 yielded varying DNA concentrations, even though the same weight of sample was used for each extraction. Due to losses of DNA during the extraction and purification process, DNA recovery is not an effective way to quantify viral concentration in samples of TM Biocontrol-1. However, in some cases, the differences in DNA recovered from lot to lot were larger than what could be reasonably be accounted for as experimental loss. This suggests that the quantity of PIBs (and virions) is not uniform from lot to lot.

3.2. Genomic variation in TM Biocontrol-1

Each of the restriction enzymes *Bgl*II, *Pst*I, and *Sal*I produced a characteristic restriction fragment length polymorphism (RFLP) profile. In this study, polymorphisms were defined as a difference of at least one fragment in the RFLP profile of the sample DNA compared with that of the MEM-75-STANDARD DNA. However, no polymorphisms were detected in any of the 51 *Op*MNPV DNA profiles produced using *Bgl*II, *Pst*I or *Sal*I to digest the 17 TM Biocontrol-1 samples.

For each restriction digest, the DNA fragment sizes were estimated by comparing the mobility of viral DNA fragments to the relative mobility of Lambda phage DNA digested with *Hin*dIII.

3.2.1. Analysis of BglII RFLPs

Digestion of TM Biocontrol-1 samples with *Bgl*II produced nine observable fragments ranging in size from 1.0 to greater than 25.5 kb (Fig. 1A). No polymorphisms were detected; all of the samples shared the same RFLP profile as the MEM positive control. The RFLP profiles in the TM Biocontrol-1 samples and the RFLP profile of the MEM-75-STANDARD DNA were consistent with previous findings in both size and number of fragments observed (Laitinen, 1995) (Table 2).

3.2.2. Analysis of PstI RFLPs

Nineteen resolvable fragments, ranging from 1.0 to 6.5 kb, were identified in the TM Biocontrol-1 samples following *Pst*I digestion (Fig. 1B). There were no observable polymorphisms in any of the TM Biocontrol-1 samples; all samples showed the same RFLP profile as that of the MEM-75-STANDARD. The *Pst*I profiles and estimated fragment sizes were consistent with results reported by Laitinen (1995), with the exception of a relatively faint 2.2 kb band noted in this study that was not observed by Laitinen (1995) (Table 2). The 2.2 kb band was also not clear in the TM Biocontrol-1 *Pst*I profile shown in Laitinen et al. (1996a), although the rest of this profile in her study matched the *Pst*I profiles produced in this study.

3.2.3. Analysis of SalI RFLPs

Digestion of *Op*MNPV DNA samples with *Sal*I produced the same electrophoretic profiles for all 17 samples and the control MEM DNA (Fig. 1C). The *Sal*I profiles contained 18 fragments ranging in size from 0.8 to 12.8 kb (Table 2). The number and molecular weights of these fragments were consistent with previously reported values (Laitinen, 1995). The *Sal*I profiles

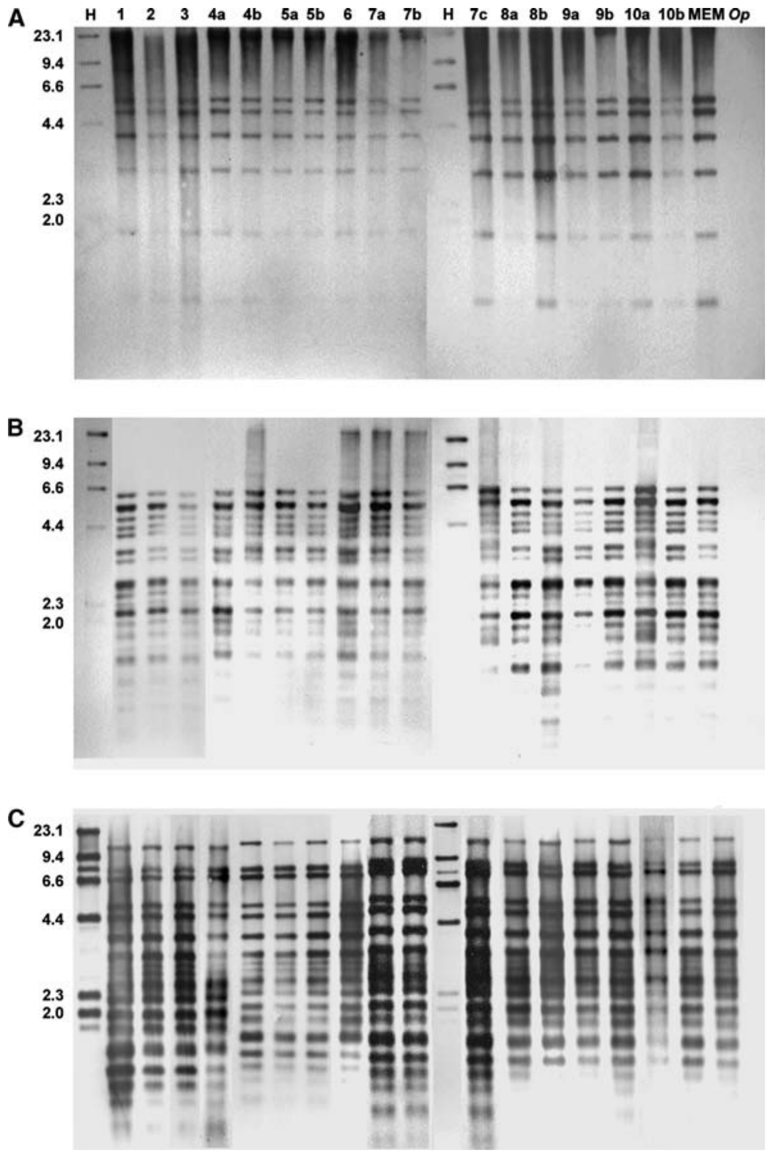

Fig. 1. (A) RFLP profiles of 17 samples of stored TM Biocontrol-1 lots digested with *Bgl*II restriction endonuclease. Values above lanes indicate lot number of the samples. (B) RFLP profiles of 17 samples of stored TM Biocontrol-1 lots digested with *Pst*I restriction endonuclease. Values above lanes indicate lot number of the samples. (C) RFLP profiles of 17 samples of stored TM Biocontrol-1 lots digested with *Sal*I restriction endonuclease. Values above lanes indicate lot number of the samples. MEM denotes MEM-75-STANDARD DNA. *Op* indicates *O. pseudotsugata* host DNA. H indicates Lambda *Hin*dIII DNA standard. Numerical values on the left indicate the molecular sizes, in kilobases, of the Lambda *Hin*dIII fragments.

reported in this study also matched the TM Biocontrol-1 *Sal*I profile shown in Laitinen et al. (1996a) and closely corresponded to the *Sal*I profile of the *Op*MNPV strain shown in Miller and Dawes (1978).

3.3. Presence of OpCPV in TM Biocontrol-1

A large, >23 kb band of *Op*MNPV DNA was observed in each successful DNA extraction from the TM Biocontrol-1 samples. In TM Biocontrol-1 samples from lots 4a, 5b, and 6, smaller weight bands were also observed in addition to this large band. These additional bands did not represent polymorphisms in the *Op*MNPV genome, because they were observed in the final DNA extract before the *Op*MNPV genome was digested. Further examination of these TM Biocontrol-1 samples indicated that in each sample there were 10 bands present ranging in size from 0.9 to 4.2 kb (Fig. 2A). The band profile very closely matched the profile described for the double-stranded RNA virus, *Op*CPV (Laitinen, 1995; Laitinen et al., 1996b) (Table 3).

To test whether the smaller bands present in the TM Biocontrol-1 samples were indeed RNA, replicate samples from lot 4a were treated with DNase I, RNase A, or with distilled water (Fig. 2B). In the replicate treated with DNase I, the smaller bands remained intact while the *Op*MNPV DNA was digested. Conversely, in the replicate treated with RNase A, the smaller bands had

Table 2
Estimated molecular weights of *Op*MNPV fragments generated by digestion with the restriction enzymes *Bgl*II, *Pst*I, and *Sal*I

Fragment	Molecular weight of digested *Op*MNPV fragments (in kb)					
	*Bgl*II		*Pst*I		*Sal*I	
	Estimated	Previously reported [a]	Estimated	Previously reported [a]	Estimated	Previously reported[a]
A	25.5	23.0+	6.5	6.5	12.8	14.5
B	24.3	23.0+	5.5	5.5	8.0	8.0
C	21.8	20.0	4.9	5.0	7.0	7.0
D	5.8	6.0	4.4	4.5	5.3	5.3
E	5.0	5.0	4.1	4.1	4.7	4.6
F	3.9	4.0	3.6	3.8	3.9	3.9
G	2.9	3.0	3.3	3.5	3.3	3.3
H	1.8	1.8	2.7	2.8	3.0	2.9
I	1.0	0.9	2.4	2.4	2.8	2.8
J			2.2	*No band reported* [b]	2.5	2.6
K			2.1	2.1	2.2	2.2
L			1.9	1.9	2.0	2.1
M			1.8	1.8	1.8	1.9
N			1.6	1.6	1.5	1.5
O			1.4	1.4	1.3	1.2
P			1.3	1.3	1.1	1.1
Q			1.2	1.2	0.9	0.9
R			1.1	1.1	0.8	0.75

[a] Reported values taken from Laitinen (1995).

[b] The 2.2 kb band observed in this study is relatively weak and was probably not observed in the profiles produced by Laitinen (1995). This 2.2 kb band is also not apparent in the *Pst*I profiles shown in Laitinen et al. (1996a).

disappeared while the *Op*MNPV genomic DNA remained intact. The replicate treated with distilled water showed both the smaller bands and the *Op*MNPV genomic DNA. The fact that RNase A digested the smaller bands while DNase I did not indicate that these bands were composed of RNA.

The electrophoretic profile of the RNA segments in samples of TM Biocontrol-1 from lots 4a, 5b, and 6 indicated that these lots were contaminated with *Op*CPV. It is not known when, where, or how the CPV contamination was introduced. However, because only 3 of the 17 TM Biocontrol-1 samples contained *Op*CPV, it is highly unlikely that the MEM-75-STANDARD used to inoculate the *O. pseudotsugata* larvae for the original production of TM Biocontrol-1 was the source of *Op*CPV. This is supported by the fact that the samples of MEM-75-STANDARD used as an experimental control in this study were also *Op*CPV free. The *Op*CPV can be considered a minor contaminant that may have been introduced inadvertently during production or processing of one lot and two sub-lots of TM Biocontrol-1.

3.4. Activity of stored product

There were no significant differences among the LD_{50} values of the samples (there was no overlap of the 95% fiducial limits) within each of the three lots. Therefore, the bioassay data of the samples within each lot were combined and the LD_{50} data were calculated for each of the three lots. Similarly, because there were no significant differences among the LD_{50} values of the fresh virus batches they were also combined and an overall LD_{50} values was calculated for the fresh *Op*MNPV samples.

3.4.1. Effect of storage time on virus efficacy

There was no significant difference in the LD_{50} values of the three lots of stored virus product (Fig. 3). The fresh virus, as expected, was the most effective (required the least number of PIBs). The stored virus lots had higher LD_{50} values, in terms of PIBs, indicating that they were about 20–30% less potent than the fresh virus (Fig. 3). However, the reduced potency or loss of efficacy of the three lots of the stored TM Biocontrol-1 were not directly related to the length of time in storage.

3.4.2. Potency ratios

The efficacy of the stored products (lots 4, 7, and 10) was also evaluated with the use of potency ratios[1] (Table 4). The relative potency provides a convenient comparison of the differences between and among samples. The overall LD_{50} of the fresh *Op*MNPV samples (potency ratio = 1.0, i.e., 100%) was compared to the overall LD_{50} of each of the 3 lots (lots 4, 7, and 10). The results show that all three lots were less potent than the fresh sample of *Op*MNPV, having potency ratios of 0.71, 0.80, and 0.68, respectively (Table 4).

[1] The relative potency of two stimuli is defined as the ratio of equally effective doses (Finney, 1971)—in our case LD_{50} values of stored virus lots and fresh virus.

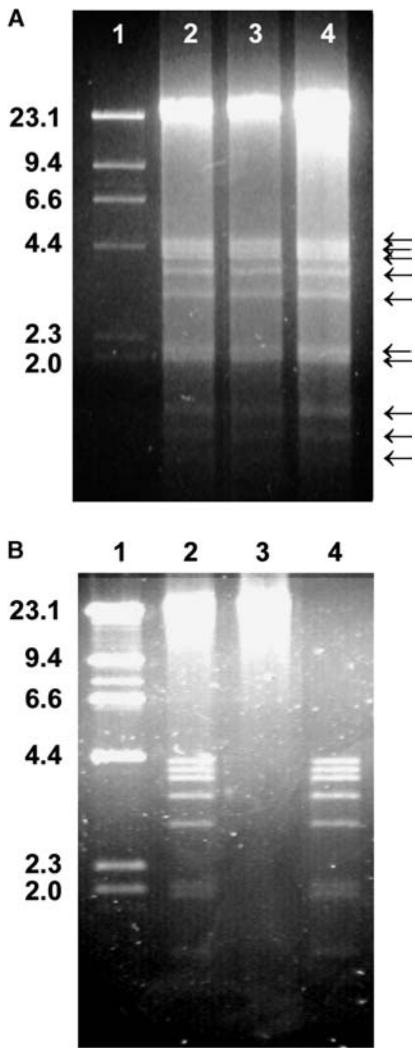

Fig. 2. (A) *Op*MNPV DNA extracts from samples of stored TM Biocontrol-1 from lot 4a (lane 2), lot 5b (lane 3), and lot 6 (lane 4) showing the presence of the contaminant, *Op*CPV. Numerical values on the left side indicate the molecular sizes of the Lambda *Hin*dIII fragments (lane 1). Arrows on the right indicate the position of the 10 RNA segments of the *Op*CPV genome. (B) RNase A and DNase I digestions of lot 4a *Op*MNPV DNA extract. Lane 1: Lambda *Hin*dIII standards. Numerical values on the left indicate size in kilobases of the segments. Lane 2: *Op*MNPV DNA extract incubated in distilled water. Both *Op*MNPV and *Op*CPV genomes remained intact. Lane 3: *Op*MNPV DNA extract incubated with RNase A. Only the *Op*MNPV genome remained intact. Lane 4: *Op*MNPV DNA extract digested with DNase I. Only *Op*CPV genomic segments remained intact.

Table 3
Molecular weight estimates, in kilobases, for putative *Op*CPV found in TM Biocontrol-1

Segment	Estimated molecular weight	Reported molecular weight[a]
1	4.2	4.3
2	4.0	4.1
3	3.8	3.6
4	3.4	3.4
5	2.9	2.9
6	2.0	2.0
7	1.9	1.95
8	1.3	1.3
9	1.1	1.2
10	0.9	1.0

[a] Reported values taken from Laitinen et al. (1996b).

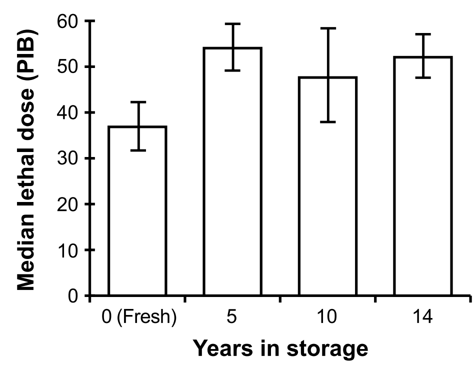

Fig. 3. Comparison of median lethal dose (LD_{50}) values of selected samples of TM Biocontrol-1, stored for 5, 10, and 14 years at −10 °C, and fresh *Op*MNPV bioassayed against newly molted third instar Douglas-fir tussock moth larvae. The larvae used in the bioassays were from the Goose Lake colony of Douglas-fir tussock moth.

Table 4
Comparison of LD_{50} values, and potency ratios of TM Biocontrol-1 samples, stored for various lengths of time at −10 °C[a] at Corvallis, OR, to those of fresh OpMNPV

Sample	Years in storage (to 2000)	LD_{50} (PIBs)	Potency ratio[b]
Lot 4	14	52.1	0.71
Lot 7	10	46.1	0.80
Lot 10	5	54.0	0.68
Fresh *Op*MNPV	0	36.9	1.00

[a] Only data from bioassays done with the Goose Lake strain were used to calculate potency ratios.

[b] Potency ratio is the ratio of the LD_{50} value for the fresh sample compared to the LD_{50} value of the TM Biocontrol-1 samples of that particular lot.

4. Discussion

*Op*MNPV genomic DNA was successfully extracted from 17 samples, representing each of the one to three package size categories of the 10 lots of TM Biocontrol-1. Viral DNA extracted from each lot was consistently observed as a single, high molecular weight band larger than 23 kb (data not shown). Digestion of *Op*MNPV genomic DNA with each of the restriction enzymes *Bgl*II, *Sal*I, and *Pst*I produced predictable and repeatable restriction fragments. All of the TM Biocontrol-1 samples yielded RFLP profiles that were consistent with the control (MEM-75-STANDARD) as well as with the reported findings (Laitinen, 1995; Laitinen et al., 1996a; Miller and Dawes, 1978). It is important to note that RFLPs can only detect base substitutions within restriction endonuclease recognition sites and relatively large DNA insertions and deletions. Although it is still possible that small, undetected changes have occurred during storage of the TM Biocontrol-1, it is highly unlikely because the *Op*MNPV virus is latent when stored at −10 °C in vacuum-sealed packages lined with aluminum foil. With no chance to replicate, the genome could not have been altered by errors in replication. There would have been no opportunity for UV-induced mutations, because the virus was stored in the dark. RFLP analysis indicates that there has been no discernible change in the *Op*MNPV DNA in any of the lots of the stored TM Biocontrol-1.

If DNA degradation had occurred in the stored TM Biocontrol-1 samples, it would have been apparent as either polymorphisms or smearing in the RFLP profiles of the samples. This was not the case, because in all of the samples *Op*MNPV DNA was observed as a single large band prior to restriction digestion. Additionally, degraded samples would have produced RFLP profiles that would have differed from the positive control (MEM-75-STANDARD) and from each other, since it is very unlikely they would have degraded at the same rate and in the same areas of the genome. Because no polymorphisms were detected in any of the stored TM Biocontrol-1 samples, it was concluded that long-term storage (up to 14 years) of the product has not affected the structure of the viral genome.

A small amount of contaminant was present in TM Biocontrol-1 samples from lot numbers 4a, 5b, and 6. The electrophoretic profile and DNase I/RNase A treatments of this contaminant agreed with previous findings of Laitinen et al. (1996b) and Galinski et al. (1982), and confirmed the identity of the contaminant as *Op*CPV. This was unexpected, although the presence of *Op*CPV in *O. pseudotsugata* has been previously noted (Laitinen et al., 1996b; Martignoni et al., 1969). It is unlikely that the presence of *Op*CPV adversely affects the potency of the product. Cypoviruses differ from nucleopolyhedroviruses in that they produce a chronic rather than lethal pathogenesis in their host (Payne and Mertens, 1983). If anything, the presence of the *Op*CPV may enhance the pathogenicity of *Op*MNPV, because the insects would have multiple infections to defend against rather than a single one. Synergistic effects have been reported between NPV and CPV (Tanada, 1956). However, further bioassays would be needed to confirm this assumption. CPV is a naturally occurring virus in field populations of *O. pseudotsugata* in some geographic locations (Galinski et al., 1994; Laitinen et al., 1996b; Williams et al., unpublished data).

There were no significant differences among the LD_{50} values of the samples either within the three lots or among the lots of TM-Biocontrol-1 stored for 5, 10 or 14 years at −10 °C. However, when the LD_{50} of these lots were compared to the LD_{50} value of the fresh virus the result of this comparison was different. When we compared the LD_{50} values of the TM Biocontrol-1 lots stored for 5, 10, and 14 years with the mean lethal LD_{50} value of the fresh virus samples an interesting trend was revealed. The LD_{50} for Lot 7 stored for 10 years was not significantly different from the LD_{50} of the fresh virus (the 95% fiducial limits overlap) but the other two lots, lot 10 and lot 4, stored for 5 and 14 years, respectively, had significantly lower efficacy, ca. 20–30% less effective than the fresh virus. A comparison of the potency ratios calculated for these stored lots confirmed this decrease in efficacy of the stored product. However, the decrease in efficacy does not appear to be correlated with the length of time in storage. A similar loss of efficacy in the same virus over a 5 year storage period was found by Martignoni (1978). Others have also reported loss of potency of insect viruses under various storage conditions over time: including the eastern hemlock looper virus (Cunningham, 1970), for the gypsy moth virus (Lewis and Rollinson, 1978), and the European spruce sawfly virus (Neilson and Elgee, 1960).

Acknowledgments

We would like to thank Drs. Eleanor White and Isabel Leal and Mr. Brett Foord for their advice and assistance in performing the DNA analyses, as well as for the use of their laboratory facilities. We thank Ann van de Raadt, Meaghan Complin, Holly Douglas, Andrea Fritz, Karolina Monge Monge, and Nicholas Conder for their technical assistance in the bioassays. We also thank Nicholas Conder for his help with the figures. Funds were jointly provided under the cooperative agreement between the USDA Forest Service, Forest Health Technology Enterprise Team, Morgantown, WV, USA and Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, Victoria, BC.

References

Cunningham, J.C., 1970. The effect of storage on the nuclear polyhedrosis virus of the eastern hemlock looper, *Lambidina fiscellaria* (Lepidoptera: Geometridae). J. Invertebr. Pathol. 16, 352–356.

Finney, D.J., 1971. Probit Analysis, third ed. Cambridge University Press, London, UK.

Fox, J., Kossobudzki, S., Fonseca da Cunha, J., 1943. Field studies on the immune response to 17-D yellow fever virus: relation to virus substrain, dose, and route of inoculation. Am. J. Hygiene 38, 113–138.

Galinski, M.S., Chow, K., Rohrmann, G., Pearson, G., Beadreau, G., 1982. Size determination of *Orgyia pseudotsugata* cytoplasmic polyhedrosis virus. Virology 123, 328–335.

Galinski, M.S., Yu, Y., Heminway, B.R., Beaudreau, G.S., 1994. Analysis of the C-polyhedrin genes from different geographical isolates of a type 5 cytoplasmic polyhedrosis virus. J. Gen. Virol. 75, 1969–1974.

Hadfield, J.S., Magelssen, R.W., 1995. TM Biocontrol-1, Production History and Inventory Status. USDA For. Serv. Region 6 Tech. Bull., Corvallis, OR.

Hughes, K.M., 1976. Notes on the nuclear polyhedrosis viruses of the tussock moth of the genus *Orgyia* (Lepidoptera). Can. Entomol. 108, 479–484.

Hughes, K.M., Addison, R.B., 1970. Two nuclear polyhedrosis viruses of Douglas-fir tussock moth. J. Invertebr. Pathol. 16, 196–204.

Kaupp, W.J., Ebling, P.M., 1990. Response of third-, fourth-, fifth-, and sixth-instar spruce budworm, *Choristoneura fumiferana* (Clem.) larvae to nuclear polyhedrosis virus. Can. Entomol. 122, 1037–1038.

Kukan, B., Otvos, I.S., Reardon, R., Ragenovich, I., 2001. Preliminary results on the efficacy of stored TM Biocontrol-1. In: Liebhold, A.M., McManus, M.L., Otvos, I.S., Fosbroke, S.L.C. (Eds.), Proceedings: Integrated Management and Dynamics of Forest Defoliating Insects. Victoria, British Columbia, Canada. August 15–19, 1999. USDA For. Serv. Gen. Tech. Rept. NE-277, pp. 74–84.

Laitinen, A., 1995. Naturally-occurring nuclear polyhedrosis virus for the biological control of Douglas-fir tussock moth, *Orgyia pseudotsugata,* McDunnough, (Lepidoptera: Lymantridae). M.Sc. Thesis, University of Victoria, Victoria, BC.

Laitinen, A.M., Otvos, I.S., Levin, D.B., 1996a. Genotypic variation among wild isolates of Douglas-fir tussock moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. J. Econ. Entomol. 89, 640–647.

Laitinen, A.M., Otvos, I.S., Levin, D.B., 1996b. Geographic distribution of cytoplamic polyhedrosis virus infection in Douglas-fir tussock moth larvae, *Orgyia pseudotsugata*, British Columbia. J. Invertebr. Pathol. 67, 229–235, doi:10.1006/jipa.1996.0037.

Lewis, F.B., Rollinson, W.D., 1978. Effect of storage on the virulence of gypsy moth nucleopolyhedrosis inclusion bodies. J. Econ. Entomol. 71, 719–722.

Martignoni, M.E., 1978. Virus in biological control: production, activity and safety. In: Brookes, M.H., Stark, R.W., Campbell, R.W. (Eds.), The Douglas-fir Tussock Moth: a Synthesis. USDA Tech. Bull. 1585, USDA For. Serv., Science and Education Agency, Washington, DC, pp. 140–147.

Martignoni, M.E., 1999. History of TM Biocontrol-1: the first registered virus-based product for control of a forest insect. Am. Entomol. 45, 30–37.

Martignoni, M.E., Iwai, P.J., Hughes, K.M., Addison, R.B., 1969. A cytoplasmic polyhedrosis virus of *Hemerocampa pseudotsugata*. J. Invertebr. Pathol. 13, 15–18.

Miller, L., Dawes, K., 1978. Restriction endonuclease analysis for the identification of Baculovirus pesticides. Appl. Environ. Microbiol. 35, 411–421.

Neilson, M.M., Elgee, D.E., 1960. The effect of storage on the virulence of a polyhedrosis virus. J. Insect Pathol. 2, 165–171.

Payne, C., Mertens, P., 1983. The cytoplasmic polyhedrosis viruses. In: Joklik, W.K. (Ed.), The Reoviridae. Plenum Press, New York, pp. 425–503.

Robertson, J.L., Preisler, H.K., 1992. Pesticide Bioassays with Arthropods. CRC Press, Boca Raton, FL.

SAS Institute, 1989–1996. SAS Users Guide: Statistics, Version 6.12. SAS Institute, Carey, NC.

Tanada, Y., 1956. Some factors affecting the susceptibility of the armyworm to virus infections. J. Econ. Entomol. 49, 52–57.

Thompson, C.G., Peterson, L.J., 1978. Rearing the Douglas-fir Tussock Moth USDA Agriculture Handbook No. 520. Combined Forestry Pest Research and Development Program, Washington, DC.